Intracellular Localization of the P21^{rho} Proteins

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Abstract. The three mammalian ras proteins associate specifically with the plasma membrane and this is essential for their biological activity. Two signals encoded within the extreme COOH terminus of the proteins specify this cellular localization; a CAAX box in combination with either a polybasic domain (p21$^{K\text{-}rasB}$) or a palmitoylation site (p21$^{Ha\text{-}ras}$ and p21$^{N\text{-}ras}$). All members of the ras-like and rho-like subfamilies of the ras superfamily of small GTP-binding proteins also have CAAX boxes with potential second site sequences resembling either p21$^{K\text{-}rasB}$ or P21$^{N\text{-}ras/Ha\text{-}ras}$. However it is not at all clear that they are each located at the plasma membrane, and in fact one of the ras-like proteins, rapl, has been localized to the Golgi (Beranger et al., 1991). None of the mammalian rho-like subfamily has yet been localized. Three forms (A, B, and C) of p21^{rho}, the prototype of this family are known; the COOH termini of p21^{rhoA} and p21^{rhoC} resemble p21$^{K\text{-}rasB}$ with a polybasic domain, whereas p21^{rhoB} resembles p21$^{N\text{-}ras/Ha\text{-}ras}$ with two cysteine residues as potential palmitoylation sites. Despite this similarity to the p21^{ras} proteins, rho proteins have been purified from both particulate and cytosolic fractions of a variety of tissues. In order to localize definitively the three rho proteins we have used an epitope tagging approach coupled to microinjection of living cells. We show that a small fraction of all three proteins is localized to the plasma membrane but the majority of p21^{rhoA} and p21^{rhoC} is cytosolic whereas p21^{rhoB} is associated with early endosomes and a pre-lysosomal compartment. Along with the results obtained with chimeric molecules using heterologous proteins attached to rho COOH termini, this suggests that the p21^{rho} proteins cycle on and off the plasma membrane and this may have important implications for their biological function.

THE p21^{rho} proteins are members of a large family of small GTP-binding proteins which share sequence homology (~30%) to p21^{ras} (Hall, 1990). They have been described in yeast, *Aplysia*, and in human cells, where three different rho genes (A, B, and C) have been found by screening a peripheral T-lymphocyte cDNA library with fragments from the *Aplysia* rho cDNA (Madaule and Axel, 1985). The proteins are expressed in all cell types so far examined (Olofsson et al., 1988). These three mammalian rho proteins are ~80% homologous to each other, and as with the three ras proteins (Ha, Ki, and N), almost all the divergence is clustered at the carboxy terminus of the protein (Yeramian et al., 1987; Chardin et al., 1988).

The biological function of p21^{rho} in mammalian cells has been studied in some detail. It was first shown that the introduction of the C3 exoenzyme from *Clostridium botulinum* into cells (which inactivates the p21^{rho} proteins by ADP-ribosylation), results in a loss of actin stress fibers followed by rounding up and loss of attachment (Chardin et al., 1989; Paterson et al., 1990). Microinjection of cells with constitutively activated p21^{rhoA}, on the other hand, led to a dramatic stimulation of actin polymerization (Paterson et al., 1990). These observations strongly suggested that the p21^{rho} proteins are involved in regulating the assembly of the actin cytoskeleton. Further and more detailed analysis of rho in fibroblasts has revealed that it is an essential component of a signal transduction pathway linking growth factor receptors to the assembly of focal adhesions and the organization of actin into stress fibers (Ridley and Hall, 1992). Although the biochemical details of this process are unknown, a number of potential regulators of p21^{rho} activity have been identified. A GTPase activating protein has been purified, and at least two nucleotide exchange proteins which are capable of stimulating (guanine nucleotide dissociation stimulator) or inhibiting (guanine nucleotide dissociation inhibitor) the dissociation of nucleotides from rho have been identified (Garrett et al., 1989; Isomura et al., 1990; Ueda et al., 1990; Fukumoto et al., 1990). It has also been demonstrated that correct COOH-terminal processing of p21^{rho} is essential for interaction with these exchange proteins (Hori et al., 1991).

Like p21^{ras}, the rho proteins each contain a carboxy-terminal CAAX box motif (C = cysteine, A = aliphatic amino acid, X = any amino acid). Since X = Leucine for all three rho proteins they would be expected to be geranylgeranylated on the cysteine residue by the enzyme geranylgeranyl transferase type I (Moores et al., 1991) and this has been directly demonstrated for p21^{rhoA} (Katayama et al., 1991). For p21^{ras}, the COOH-terminal CAAX box along with a polybasic region (p21$^{K\text{-}ras}$) or upstream palmitoylation site (p21$^{Ha\text{-}ras}$, p21$^{N\text{-}ras}$) has been shown to lead to plasma membrane localization (Hancock et al., 1991). The presence of similar polybasic regions in p21$^{rhoA/rhoC}$ and a potential palmitoylation site in p21^{rhoB} strongly suggests that the p21^{rho} proteins would localize to the plasma membrane

in an analogous fashion to $p21^{ras}$. However *rho* gene products have been purified from a variety of cellular compartments in mammalian tissues. First, $p21^{rhoB}$ and subsequently $p21^{rhoA}$ were purified from a bovine brain membrane fraction, whereas $p21^{rhoA}$ has also been purified from bovine adrenal gland cytosol and aortic smooth muscle cytosol (Kawahara et al., 1990; Yamamoto et al., 1988; Hoshijima et al., 1990; Narumiya et al., 1988; Braun et al., 1989). There have been reports that both $p21^{rhoB}$ and $p21^{rhoA}$ are located in synaptic plasma membranes and that two small GTP-binding proteins which are substrates for the C3 exoenzyme are present in a rat liver Golgi-enriched subcellular fraction (Isomura et al., 1991; Kim et al., 1989; Toki et al., 1989). The above studies clearly indicate that the cellular localization of the three $p21^{rho}$ proteins may be different from each other and from $p21^{ras}$ which to date has only been found in the plasma membrane (Willingham et al., 1980; Hancock et al., 1990). We have therefore used a microinjection approach with epitope-tagged rho expression vectors to analyze the localization of rho A, B, and C in a variety of cell types.

Materials and Methods

Materials

Oligonucleotide-directed mutagenesis system was obtained from Amersham International (Amersham, UK), Keyhole Limpet Hemocyanin was obtained from Calbiochem (Nottingham, UK), rhodamine/fluorescein-linked second antibodies and reactigel HW-65 were obtained from Pierce and Warriner (Chester, UK), and synthetic peptides were synthesized by Multiple Peptide Systems (San Diego, CA). All other chemicals were obtained from Sigma Chemical Co. (Poole, UK). Hybridoma cells expressing the 9E10 anti-myctag mouse mAb were a gift from G. Evan (Imperial Cancer Research Fund, London, UK). A protein A fusion construct containing the 17 COOH-terminal residues of $p21^{K-ras(B)}$ was a gift from C. J. Marshall (Institute of Cancer Research, London). Fluorescent-labeled transferrin and antimannose-6-phosphate receptor antibody were kind gifts from Colin Hopkins (University College, London) and G. Sahagian (Yale University, New Haven, CT). Rabbit anti-rab6 antibody was a kind gift from A. Tavitian (Inserm, Paris).

DNA Constructs

A previously described eukaryotic expression vector containing rhoA cDNA under the control of the SV-40 early promoter (pEXVrhoA) was cut at the natural *AccIII* site at codon 3 and ligated to a double-stranded oligonucleotide encoding the peptide sequence MEQKLISEEDL to create an epitope tag at the amino terminus (Paterson et al., 1990). This sequence is the epitope for the 9E10 anti-myc mAb (Evan et al., 1985). An analogous construct containing pEXVmyc.rhoB and pEXVmyc.rhoC was also constructed. The myctag epitope was also added to the NH_2 terminus of pEXVHa-ras. Myc-tagged pEXVrab6 was a gift from J. Hancock (Royal Free Hospital, London). Plasmids encoding the rhoA and rhoB cDNAs lacking the NH_2-terminal myc-tag sequence were also constructed in pEXV. Oligonucleotide-directed mutagenesis was used to introduce (*a*) *Asp700* sites into both rhoA and rhoB sequences at codon 137, and (*b*) a *PstI* site at codon 178 in the rhoA sequence (rhoB has a natural *PstI* site at codon 178). These restriction sites were used to generate chimeric cDNAs containing rhoA and rhoB sequences. Oligonucleotide-directed mutagenesis was also used to incorporate point mutations into both pEXVmycrhoA and pEXVmycrhoB. A mammalian expression vector (pCDM8) containing the coding region of *Staphylococcus aureus* protein A under the control of the cytomegalovirus promotor, and which encodes *SmaI*, *BamHI*, *SalI*, *EcoRI*, and *PstI* within the extreme COOH terminus of the protein A coding region were kind gifts from A. Ashworth (Institute of Cancer Research, London) (Lowenadler et al., 1986). These vectors were used to make fusion cDNAs between protein A and rho sequences. Two fragments from both rhoA and rhoB were subcloned into the protein A–containing vector. These fragments were from the *PvuII* site at codon 60 (blunt end ligated into an *EcoRI* site), and from the *PstI* site at codon 178 (blunt end ligated into a *SmaI* site). COOH-terminal fragments of both rhoA and rhoB containing 133 and 136 residues respectively were subcloned into a previously described plasmid pEXV N-ras at the common *PvuII* site at codon 60 to create ras/rho chimeras (Hancock et al., 1988).

Nuclear Microinjection of Cells with Mammalian Expression Vectors

MDCK and Rat-2 cells were grown in DME containing 10% FCS at 37°C, in an atmosphere of 90% air and 10% CO_2. MDCK and Rat-2 cells were seeded onto marked areas of 13-mm glass coverslips 1–3 d before injection. Purified DNAs (50 μg/ml) were microinjected into cell nuclei (Paterson et al., 1990). After incubation at 37°C for 5–20 h, cells were immunolabeled for the presence of rho proteins or protein A–rho fusion proteins.

Antibodies to $P21^{rhoA}$ and $P21^{rhoB}$

The peptides NH_2CNDEHTRRELAKMKQE and NH_2CSDEHVRTELARMKQE correspond to residues 119–132 in rhoA and rhoB, respectively. NH_2-terminal cysteine residues were added to conjugate the peptides to Keyhole Limpet Hemocyanin. Rabbits were injected four times with 1 mg of conjugated peptide over a 4-wk period and the serum was obtained after 5 wk. Serum was loaded onto a reactigel HW65-peptide column and the column washed with 0.1 M sodium phosphate buffer, pH 8.0. Antibody was eluted with 3.5 M potassium thiocyanate and 0.5 M Ammonia, pH 11.2. Fractions (0.5 ml) were collected into 1 M ice-cold sodium phosphate buffer, pH 7.0 (0.5 ml), and antibody-containing fractions were pooled, dialyzed against PBS, and concentrated to ~2 mg/ml in a centricon 10 microconcentrator (Amicon, Beverly, MA).

Immunofluorescence

Cells were washed twice with PBS, pH 7.2 (PBSA), and fixed for 10 min at room temperature in 3% paraformaldehyde in PBSA. After twice washing in PBS, cells were incubated for 10 min with 50 mM ammonium chloride in PBSA. Cells were then washed once and permeabilized with PBSA containing 0.2% Triton X-100 for 10 min at room temperature, and finally washed twice. Anti-myctag staining was performed by incubation for 1 h with 1:400 9E10 anti-myctag mAb (ascites fluid diluted in PBSA). Cells were then washed six times and incubated for 1 h at room temperature with 1:200 fluorescein-conjugated anti–mouse antibody (Pierce Chemical Co., Rockford, IL) in PBSA. After washing six times, coverslips were inverted and mounted on glass microscope slides using moviol mountant containing *p*-phenylenediamine (0.1%) antiquenching agent. Slides were then viewed on a MRC-600 confocal imaging system equipped with a Krypton-Argon laser. Anti–protein A staining was achieved using a 1-h incubation at room temperature, with 7F7 mouse monoclonal (1:1,000 in PBSA) as the primary antibody (Shultz et al., 1988). When staining was performed with anti-$p21^{rhoA}$ and anti-$p21^{rhoB}$ polyclonal antibodies, cells were fixed and permeabilized as for the 9E10 anti-myctag antibody, except prior to incubation with polyclonal antibodies, cells were incubated in 10% FCS in PBSA for 30 min. Peptide-purified polyclonal antibodies were used for 1 h at room temperature at 1:50 dilution in an incubation buffer containing 50 mM Tris-HCl, pH 7.6; 1% NP-40; 0.5% sodium deoxycholate, and 5 mM EDTA. After washing, cells were treated with fluorescein-conjugated goat anti–rabbit antibody (Pierce Chemical Co.) diluted 1:2,000 in incubation buffer. Cells were then washed six times in incubation buffer, twice in PBSA, and mounted as described for the 9E10 antibody. For rab6 staining, fixed cells were permeabilized in 0.5% saponin/0.2% BSA in PBS instead of Triton X-100 and all subsequent antibody incubations and washings were done in the presence of saponin. Lysosomes were visualized in injected and noninjected cells by incubating cells with Lucifer yellow (1 mg/ml) in DME for 6 h at 37°C followed by a 12-h chase in DME. Immunofluorescent visualization of the mannose-6-phosphate receptor was achieved in an identical manner to that of the 9E10 antibody using a polyclonal antimannose-6-phosphate receptor antibody (1:200 dilution in PBSA). Early endosomes were observed by allowing cells to take up FITC-transferrin in culture (100 μg/ml in DME) for 16 h. After uptake cells were washed 3 times in PBSA, fixed in 3% paraformaldehyde, and further processed for other immunochemical stains. For triple labeling FITC-transferrin, rabbit antimannose-6-phosphate receptor antibody labeled by a donkey anti–rabbit antibody conjugated with the Cy5 fluorophore, and the mouse 9E10 antibody labeled by a donkey anti–mouse antibody conjugated with the rhodamine–lissamine fluorophore were used.

Transfection and Fractionation of COS-1 Cells

COS-1 cells were transfected with 10 μg plasmid DNA per 10-cm^2 petri dish using a DEAE-dextran method as described previously (Lowe and Goeddel, 1987). After transfection, cells were grown for 48 h after which they were harvested and fractionated as previously described (Cales et al., 1988) except that membranes were pelleted at 120,000 *g* for 30 min to produce P100 pellets and S100 supernatants.

Western Blotting

Samples were separated on a 15% SDS-polyacrylamide gel and electrotransferred to nitrocellulose (BA 85, Schleicher & Schuell, Inc., Keene, NH) overnight at 20 mA at 4°C. The blot was blocked with 5% dried milk powder, 10 mM Tris, pH 7.5, 150mM NaCl, 0.05% Tween 20 (TBST) for 1 h, 9E10 anti-myctag antibody (1:1,000 ascites fluid) was added and incubated for an additional 1 h at room temperature. After five washes in TBST, the blot was incubated with anti-mouse HRP (Pierce Chemical Co., 1:10,000) for 1 h at room temperature, washed five times in TBST, and developed with an enhanced chemiluminescence Western blotting detection system (Amersham International).

Results

Nuclear microinjection of cDNA expression vectors encoding p21^{rho} proteins with or without the myctag epitope resulted in morphological changes (rho-phenotype) and actin polymerization as previously reported (Paterson et al., 1990). The onset of this effect could be observed by 5 h after injection with either plasmid, showing that addition of 10 amino acids to the NH_2 terminus of rho does not effect its biological activity. MDCK cells were used principally for detection of plasma membrane-associated fluorescence, whereas rat-2 fibroblasts were chosen to observe intracellular structures.

Localization of Rho A, B, and C

After nuclear microinjection and staining with the 9E10 anti-myctag antibody (14–16 h after microinjection) myc.rhoA and myc.rhoC showed an identical pattern of fluorescence in rat-2 cells; namely a floccular staining throughout the cytoplasm with a small proportion located at the plasma membrane (Fig. 1, *c* and *d*). At later times (18–20 h) and as the cells began to adopt a rho morphological phenotype, an increasing amount of the protein was found associated with the plasma membrane. When rhoA and rhoC were expressed in MDCK cells the staining was again principally cytosolic. To confirm the cytosolic nature of rhoA and rhoC proteins, goat gamma globulin (1 mg/ml) was microinjected into rat-2 cells which had 11 h previously been injected with expression plasmids encoding the rhoA or rhoC proteins. After a further 1-h incubation the cells were fixed and costained for goat gamma globulin using anti-goat-FITC, and myc-tagged proteins using 9E10 and anti-mouse TRITC. The proteins showed identical patterns of fluorescence (Fig. 2, *a* and *b*) and we conclude that rhoA and rhoC are primarily cytosolic but with a small proportion at the plasma membrane.

Myc.rhoB on the other hand gave a very different pattern of fluorescence when expressed in cells. Staining was predominantly associated with cytoplasmic vesicles typically clustered around the nucleus and becoming fewer in number toward the periphery of the cell (Fig. 1 *b*). These vesicles were present in both Rat-2 and MDCK cells and in a variety of other cell types including Swiss 3T3, NIH 3T3, COS-1, and rat embryo fibroblasts. As with myc.rhoA and myc.rhoC, a small proportion of myc.rhoB was also located at the plasma membrane. As in the case of rhoA and rhoC there was only a small amount of plasma membrane staining visible at early time points which increased as the cells developed a rho morphology.

To demonstrate that the addition of the NH_2-terminal myctag sequence had no effect on the localization of rho proteins, constructs lacking the myctag sequence were injected and stained with either anti-rhoA or anti-rhoB antipeptide antibodies. The staining patterns observed in these experiments were essentially the same as those obtained with the 9E10 anti-myctag antibody although nonspecific background fluorescence was higher (data not shown). As a further control the myctag sequence was attached to the NH_2 terminus of Ha-ras and this did not effect its localization, which was almost exclusively at the plasma membrane (Fig. 1 *a*). Similarly injection of a myc-rab6 plasmid followed by staining for the myctag produced similar Golgi staining to endogenous rab6 visualized with anti-rab6 antibody (Fig. 1, *e* and *f*) (Goud et al., 1990). It is particularly important to rule out that the observed localizations were not due to high levels of expression of these proteins. Since we were not able to observe endogenous rho proteins using our anti-rho antibodies, we addressed this potential problem by carrying out fluorescent labeling at different times after injection of DNA. Even at the earliest times, when tagged rhoA or rhoB could only just be observed, the localization was identical to that shown in Fig. 1. Overexpression of the myctag-rab6 plasmid, on the other hand, clearly saturated its normal localization site and produced cytosolic staining (Fig. 1 *f*, *arrows*).

To confirm that rhoA and rhoC are cytosolic while rhoB is attached to a membranous compartment, we expressed the plasmids transiently in COS-1 cells. Separation of cell homogenates into total membranes (P100) and cytosol (S100), showed that myc.Ha-ras and myc.rhoB were associated with the P100 fraction whereas both myc.rhoA and myc.rhoC associated almost exclusively with the cytosolic fraction (Fig. 3). Overdevelopment of Western blots showed small amounts of rhoA and rhoC in the P100 fraction consistent with the amount of protein observed in the plasma membrane by immunofluorescence. The presence of rhoB and ras in the S100 fraction is a consequence of high level expression in COS-1 cells and has previously been observed in similar experiments with ras (Hancock et al., 1989).

Colocalization of RhoB with Subcellular Structures

To examine the localization of the mycrhoB in more detail, endosomes and lysosomes were visualized in rat-2 cells by incubation overnight in Lucifer yellow (1 mg/ml), which enters the cell by endocytosis and equilibrates throughout the endocytotic pathway (Swanson et al., 1985). Lucifer yellow was then chased using a 12-h incubation in media without Lucifer yellow so as to label predominantly mature lysosomes (Swanson et al., 1987; Griffiths et al., 1989). When this analysis was carried out on cells that had been microinjected with a mycrhoB-containing plasmid no significant overlap in fluorescence was observed (Fig. 4, *a* and *b*). To examine early endosomes, transferrin labeled with fluorescein was incubated with cells for 16 h at 37°C. This procedure is known to label predominantly early endosomes (Hopkins, 1983). When rhoB-injected rat-2 cells were incubated with FITC-transferrin a significant overlap in fluores-

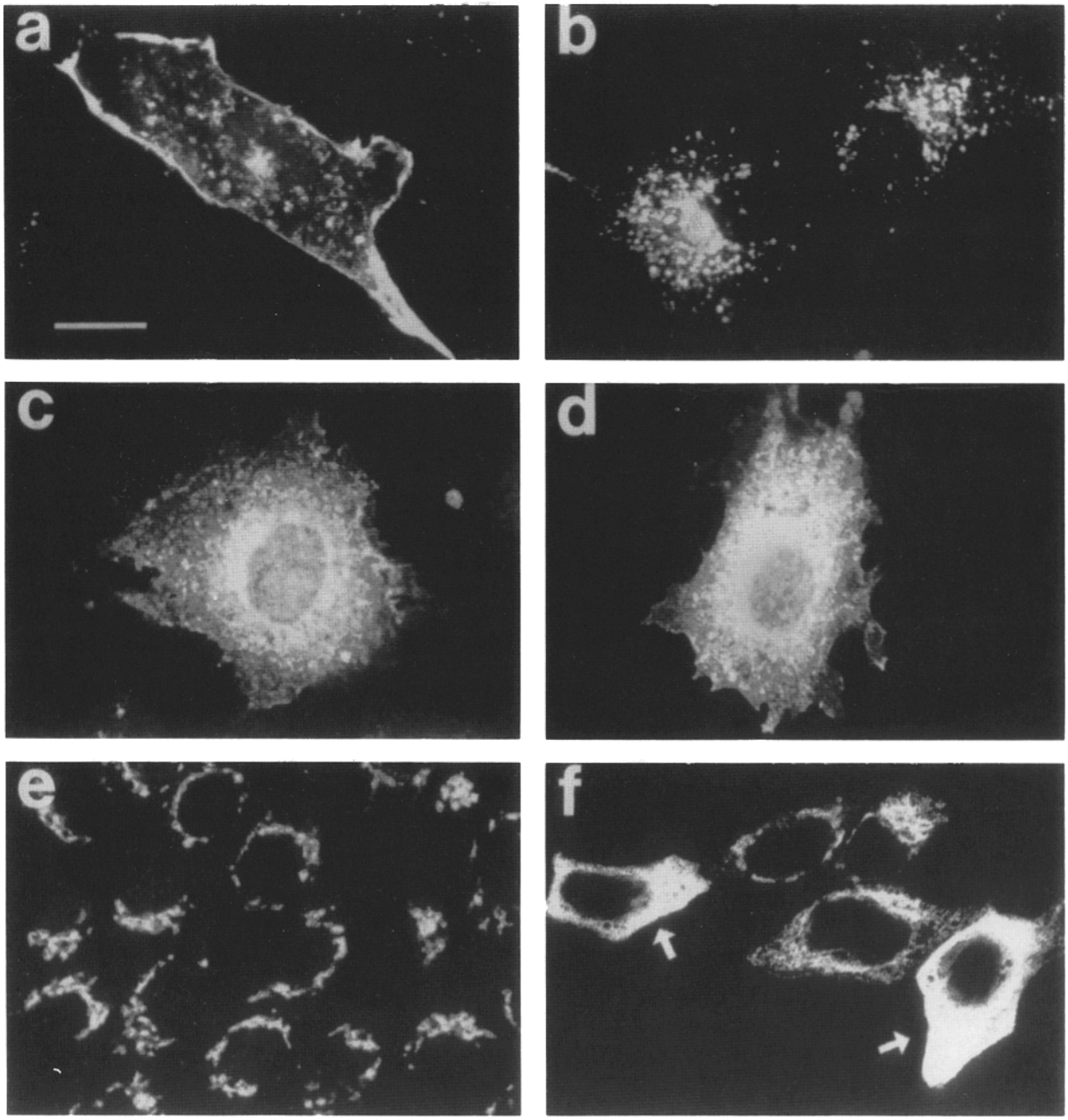

Figure 1. Localization of rho proteins in microinjected cells. Rat-2 cells (*a*, *b*, *c*, and *d*) or MDCK cells (*f*) stained with 9E10 mouse monoclonal anti-myctag antibody 14 h after injection with pEXV plasmid DNA (50 μg/ml) encoding cDNAs of (*a*) p21myc$^{Ha\text{-}ras}$, (*b*) p21$^{myc.rhoB}$, (*c*) p21$^{myc.rhoA}$, (*d*) p21$^{myc.rhoC}$, and (*f*) mycrab6. Endogenous rab6 localized in MDCK cells using anti-rab6 antibody (*e*). Arrows in (*f*) indicate cells where rab6 is mislocalized due to high level expression. Bar, 10 μm.

cence could be clearly observed (Fig. 4, *c* and *d*). Almost all transferrin positive vesicles were seen to be rhoB positive though there were many rhoB positive vesicles particularly close to the nucleus, which were negative for transferrin. Note that in the fields shown in Fig. 4, *a*, *c*, and *e* only one cell has been microinjected and is expressing rhoB, whereas all cells in the fields shown in Fig. 4, *b*, *d*, and *f* are being visualized. To further characterize this compartment, cells microinjected with rhoB DNA were costained for rhoB and for the mannose-6-phosphate receptor (Sahagian and Neufeld, 1983; Stoorvogel et al., 1989). There was significant colocalization of the two proteins, particularly close to the nucleus, though again overlap between the two fluorescence patterns was not complete with many rhoB positive vesicles not staining positive for mannose-6-phosphate receptor (Fig. 4, *e* and *f*). Finally the use of a triple labeling procedure allowed us to localize rhoB, mannose-6-phosphate receptor, and transferrin in the same cell (Fig. 5). It can be seen that merging the mannose-6-phosphate receptor and transferrin fluorescence images (Fig. 5 *d*) produces an almost identical

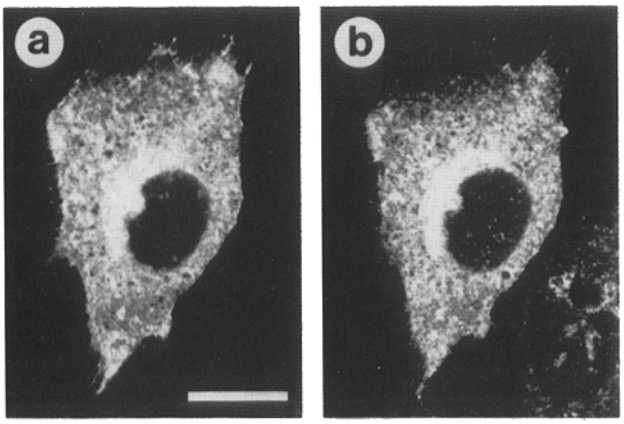

Figure 2. Cytosolic localization of rhoA. (*a*) Rat-2 cell injected with pEXVrhoB plasmid DNA (50 μg/ml) and after 12 h fixed and stained with 9E10 mouse monoclonal anti-myctag antibody and (*b*) the same cell injected 1 h before fixation with goat gamma globulin (1 mg/ml) and stained with antigoat antibody. Bar, 10 μm.

pattern of fluorescence to that observed with rhoB (Fig. 5 *a*). We conclude that rhoB is situated on both early endosomes and on a prelysosomal compartment but not on mature lysosomes. Essentially the same results are found in COS-1 and MDCK cells.

Protein A–Rho Fusion Proteins

In an attempt to identify specific sequences within the rho structure that are responsible for the very different cellular localizations of myc.rhoA and myc.rhoB, protein A fusion proteins were constructed carrying rhoA or rhoB COOH-terminal fragments (14 or 17 residues, respectively). These short fragments were chosen since recent studies using the 17 COOH-terminal residues of $p21^{K\text{-}ras(B)}$ or the 10 COOH-terminal residues of $p21^{Ha\text{-}ras}$ demonstrated that these sequences could function as plasma membrane targeting signals for a heterologous protein when introduced into COS-1 cells or injected into MDCK cells (Hancock et al., 1991). Two other fragments of the $p21^{rho}$ proteins represented the last 133 residues of rhoA and the last 136 residues of rhoB were also fused to protein A. These larger fragments were used since $p21^{rhoA}$ and $p21^{rhoB}$ are almost identical in the remaining NH_2-terminal 60 residues.

Analysis of fluorescence obtained with 7F7 mAb, which

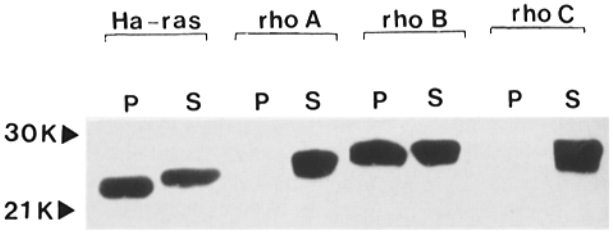

Figure 3. Membrane and cytosol fractionation of rho proteins. Western blot with 9E10 anti-myctag antibody on cell lysates of COS-1 transfected with EXV.mycHa-ras, EXVmyc.rhoA, EXV-myc.rhoB, and EXVmyc.rhoC which have been fractionated into P100 (*P*) and S100 (*S*) by ultracentrifugation.

will selectively recognize the protein A part of the fusion, showed that the protein A with no COOH-terminal rho sequence was expressed and localized in the cytosol within MDCK cells (Fig. 6 *c*), with a proportion appearing in the nucleus. The fusion protein containing the COOH-terminal 17 residues of rhoB was found almost exclusively at the plasma membrane in MDCK cells (Fig. 6 *b*), identical to the localization of a protein A–fusion containing the COOH-terminal sequence of $p21^{k\text{-}ras(B)}$ in these cells (Fig. 6 *a*). Although the analogous rhoA construct showed plasma membrane fluorescence a significant amount was still found in the cytosol (Fig. 6 *d*). Interestingly the larger protein A–fusion containing residues 60–196 of $p21^{rhoB}$ was also confined to the plasma membrane with none of the vesicular staining observed with full-length rhoB (data not shown). We conclude from this experiment that either the NH_2-terminal 60 amino acids of the rhoB protein must be essential for its cellular localization or that correct folding of the rho proteins is necessary.

N-ras-rho Chimeras

To test if the NH_2-terminal 60 amino acids of rho are required for correct localization we constructed chimeric proteins where the NH_2-terminal 60 residues of both rhoA and rhoB were substituted by those of normal N-ras. These chimeras encode the ras effector sequences (McCormick, 1989 for review) and in fact these N-ras-rho constructs are able to transform NIH3T3 cells almost as efficiently as oncogenic N-ras (data not shown). The chimeric proteins contain the epitopes to which the rabbit antipeptide antibodies from rhoA and rhoB were made and they could be localized in both MDCK and rat-2 cells using these antibodies. The N-ras control protein was visualized in these cells using Y13-259 anti-ras rat mAb (data not shown). The N-ras chimeric protein carrying the last 136 residues of rhoB localized almost exclusively to the plasma membrane in both MDCK and rat-2 cells suggesting that sequences within the first 60 amino acids of rhoB are required for correct localization to endosomes and prelysosomes. Injection of MDCK cells with the N-ras chimeric construct carrying the COOH-terminal 133 residues of rhoA showed both a plasma membrane and cytosolic fluorescence similar to the protein A–fusion protein carrying the same fragment (data not shown).

RhoA/RhoB Chimeras

To identify further sequences responsible for the different localization of rhoA and rhoB we constructed rhoA/B chimeras. First rhoA (residues 1–178) was attached to the last 17 residues of rhoB (residues 179–196), or rhoA (residues 1–137) was ligated to the last 59 residues of rhoB (residues 138–196). When the constructs were injected into either rat-2 or MDCK cells and stained with the 9E10 anti-myctag antibody they both produced a vesicular fluorescence pattern identical to that of full-length myc.rhoB (Fig. 7, *b* and *d*). The reciprocal constructs containing 14 or 56 residue COOH-terminal fragments of rhoA attached to myc.rhoB both produced a pattern of fluorescence identical to that of full-length myc.rhoA (Fig. 7, *a* and *c*). All chimeric proteins appeared to be biologically active based on their effect on cell shape and actin polymerization. We conclude that the

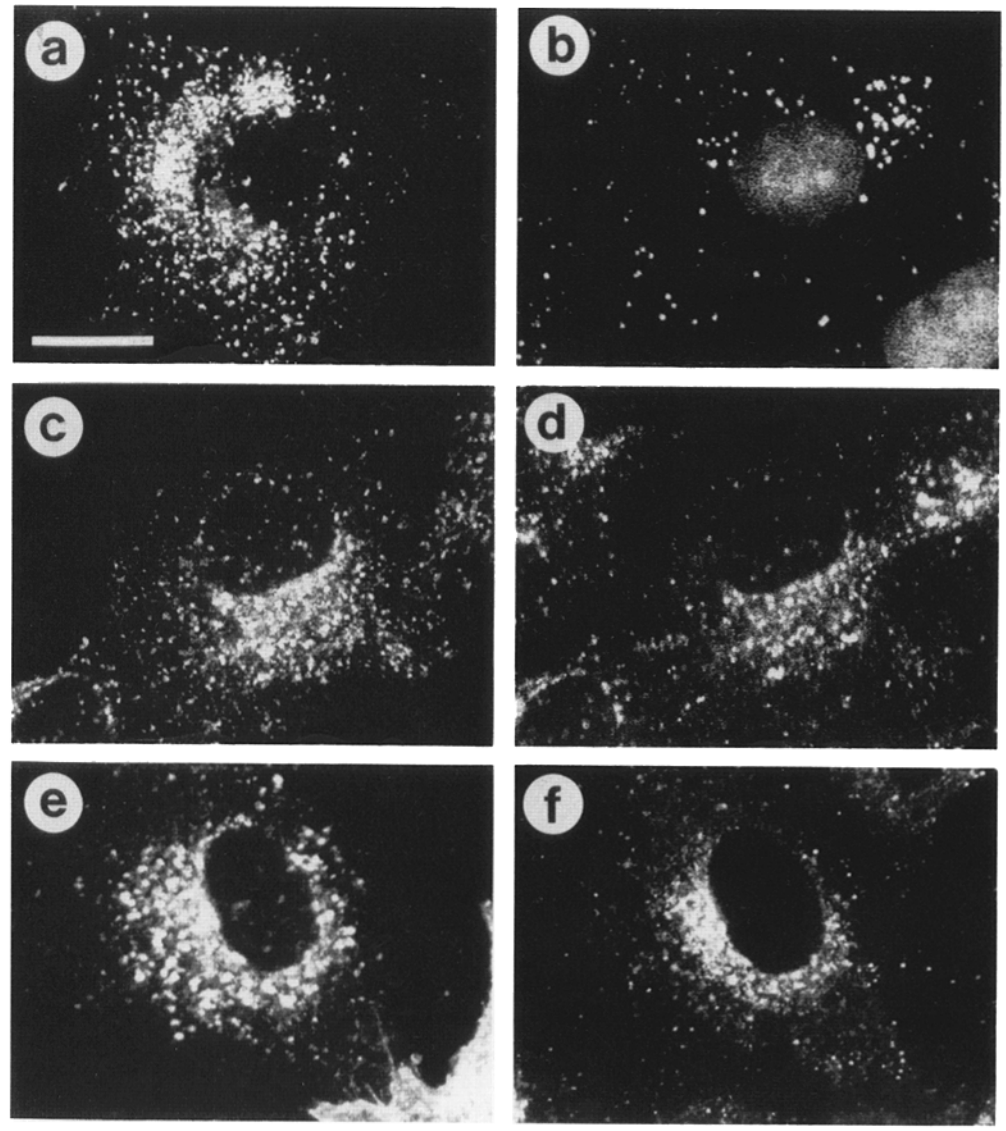

Figure 4. Intracellular localization of rhoB. Rat-2 fibroblasts injected with pESVmyc rhoB plasmid DNA (50 μg/ml) and incubated overnight in DME supplemented with 10% FCS and stained (*a*, *c*, and *e*) with 9E10 anti-myctag antibody 16 h after injection. (*b*) cells in *a* were also incubated with Lucifer yellow (1 mg/ml) for 6 h followed by chase for 12 h, (*d*) cells in *c* were also incubated with FITC labeled transferrin. (*f*) Cells in *e* were also stained with antimannose-6-phosphate receptor antibody. Bar, 10 μm.

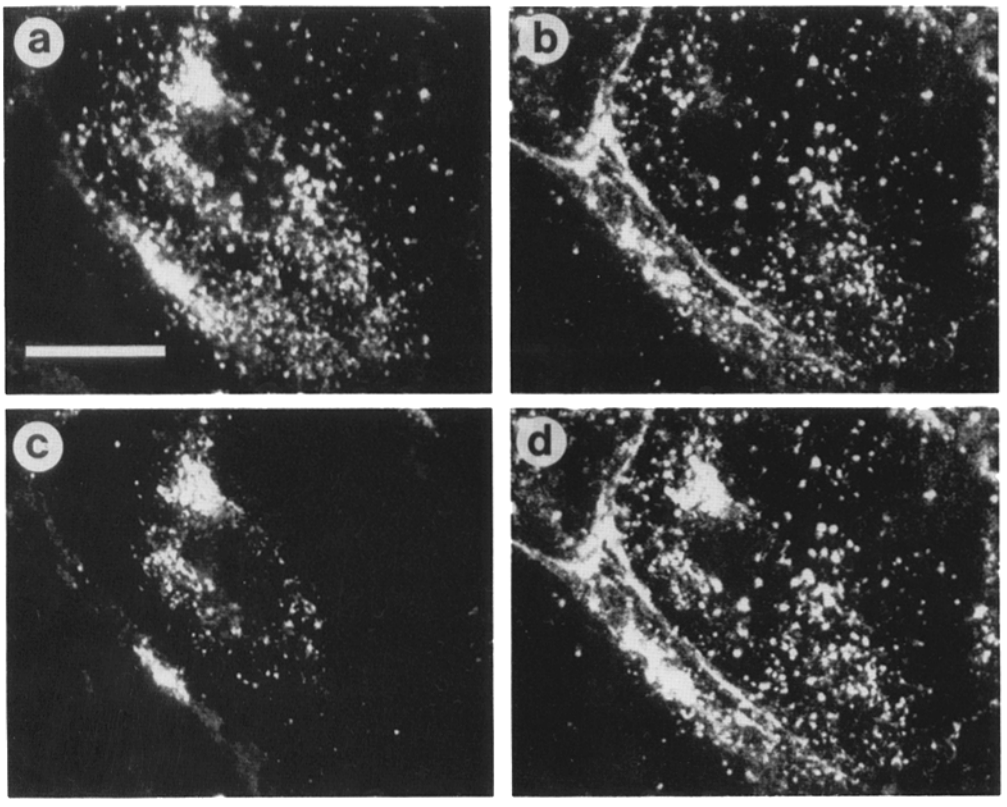

Figure 5. Triple labeling of cells for rhoB, transferrin, and mannose-6-phosphate receptor. A rat-2 fibroblast injected with pEXVmyc rhoB plasmid DNA (50 μg/ml) and incubated overnight in DME supplemented with 10% FCS and 100 μg/ml transferrin–FITC and stained 16 h after injection with 9E10 anti-myctag antibody and antimannose-6-phosphate receptor antibody. (*a*) 9E10 antibody showing rhoB localization, (*b*) mannose-6-phosphate receptor antibody, (*c*) transferrin, (*d*) superimposed (merged) images of fluorescence patterns obtained from transferrin and mannose-6-phosphate receptor. Note: in *b* and *d* a process from a neighboring cell not injected with rhoB DNA can be seen. Bar, 10 μm.

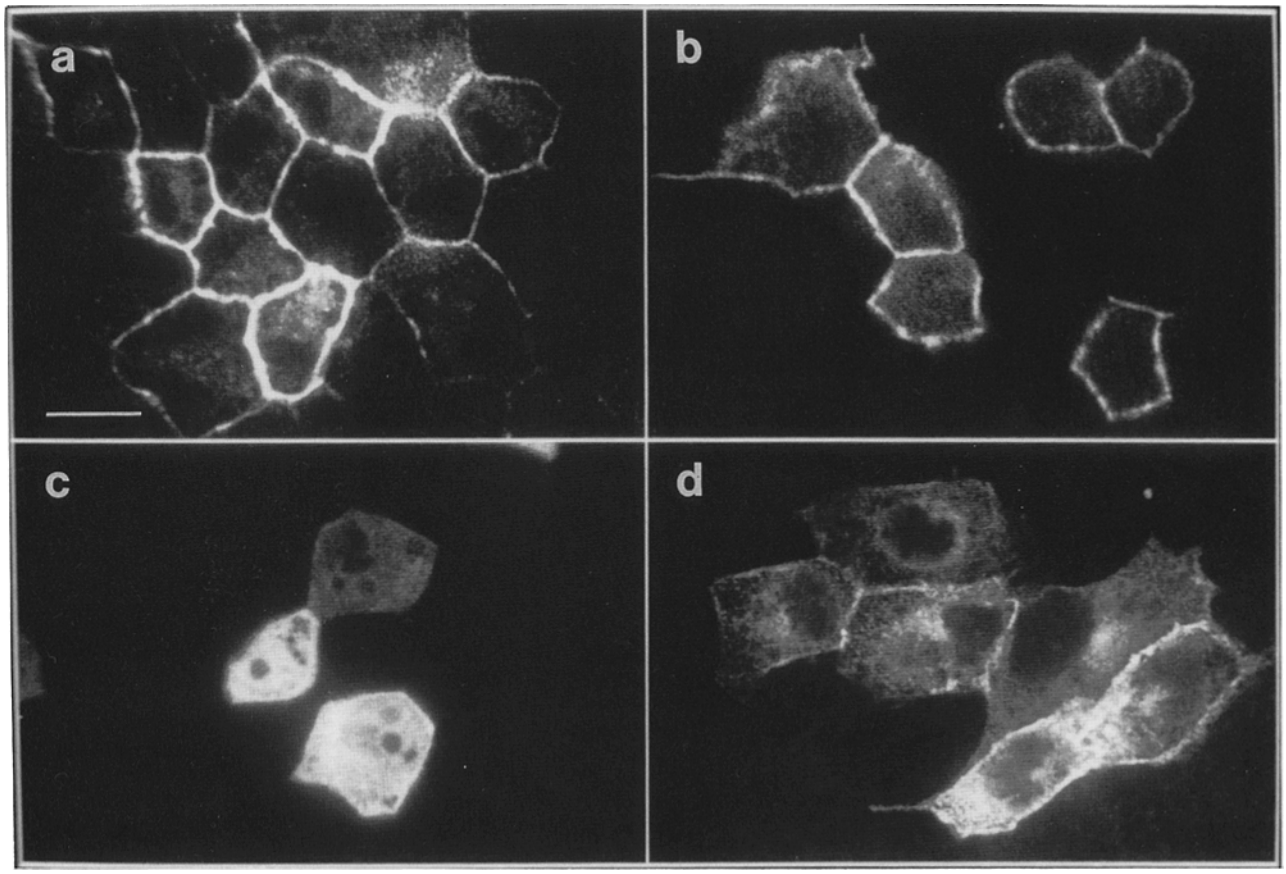

Figure 6. Localization of protein A/rho chimeric proteins. MDCK cells microinjected with protein A–fusion constructs in pCDM8 and stained after 16 h with 7F7 mouse mAb and visualized using a rabbit fluorescein-conjugated anti-mouse antibody. (*a*) Protein A with 17 COOH-terminal residues of $p21^{k\text{-}ras(B)}$ comprising SKDGKKKKKKSKTKCVIM. (*b*) Protein A with 18 COOH-terminal residues of $p21^{rhoB}$ comprising LQKRYGSQNGCINCCKVL. (*c*) Protein A with no additional COOH-terminal sequence. (*d*) Protein A with 15 COOH-terminal residues of $p21^{rhoA}$ comprising LQARRGKKKSGCLVL. Bar, 10 μm.

signal for the different localization of rhoA and rhoB resides in the COOH-terminal 17 residues but that this is only effective in combination with a functional rho protein.

Effects of COOH-terminal Mutations on Localization

Substitution of the cysteine residue for serine at codon 190 in the myc.rhoA CAAX box (CLVL→SLVL) resulted in a marked reduction of the normal cytosolic fluorescence, instead producing a strong nuclear localization in MDCK cells. A similar localization has also been observed for the three ras proteins mutated in their CAAX box, which when overexpressed accumulates in the cytosol, and more predominantly in the nucleus (Hancock et al., 1990). A mutation in the CAAX box of myc.rhoB (CKVL→SKVL), in which the residue at codon 193 was substituted from a cysteine to a serine, resulted in cytosolic and nuclear fluorescence in MDCK cells (Fig. 8 *b*), again similar to that of the cysteine to serine CAAX mutated ras proteins. RhoB has an additional cysteine at the carboxy terminus (i.e., CCKVL), mutation of this upstream cysteine (CCKVL→SCKVL) produced a distinct pattern of fluorescence from that of the wild-type protein (Fig. 8 *c*). In this case the protein was absent from the early endosomal compartment but instead associated with a number of tubulo-vesicular structures which were located around the perinuclear region of the cell. The identity of this compartment is not known, but it was not observed with either the transferrin or mannose-6-phosphate receptor markers. Mutation of a third upstream cysteine (to serine) at codon 189, a potential palmitoylation site in rhoB based on the sequence similarity to $p21^{Ha\text{-}ras}$, had little apparent effect on the localization of rhoB and was observed on prelysosomal/endosomal vesicles (Fig. 8 *d*). Other mutations in the COOH terminus of myc.rhoB in which codons 183 and 194 were mutated from tyrosine and lysine to arginine and leucine, respectively, were also without effect on the subcellular localization of the protein and were fully biologically active on cell morphology and actin polymerization.

Discussion

The three $p21^{rho}$ proteins have been purified from both cytosolic and particulate fractions of cell extracts by a number of groups and their exact in vivo localizations are unclear. Microinjection of tagged-rho cDNAs in an SV-40 based expression vector has allowed us to localize both rhoA and rhoC predominantly to the cytosol with a small fraction at the plasma membrane. This localization pattern was observed in a variety of different cell types and at a range of expression levels and is consistent with published reports of rhoA purification from cytosolic extracts of adrenal gland and aortic smooth muscle (Katayama et al., 1991; Kawahara et al., 1990). RHO1, the yeast homologue of the mammalian

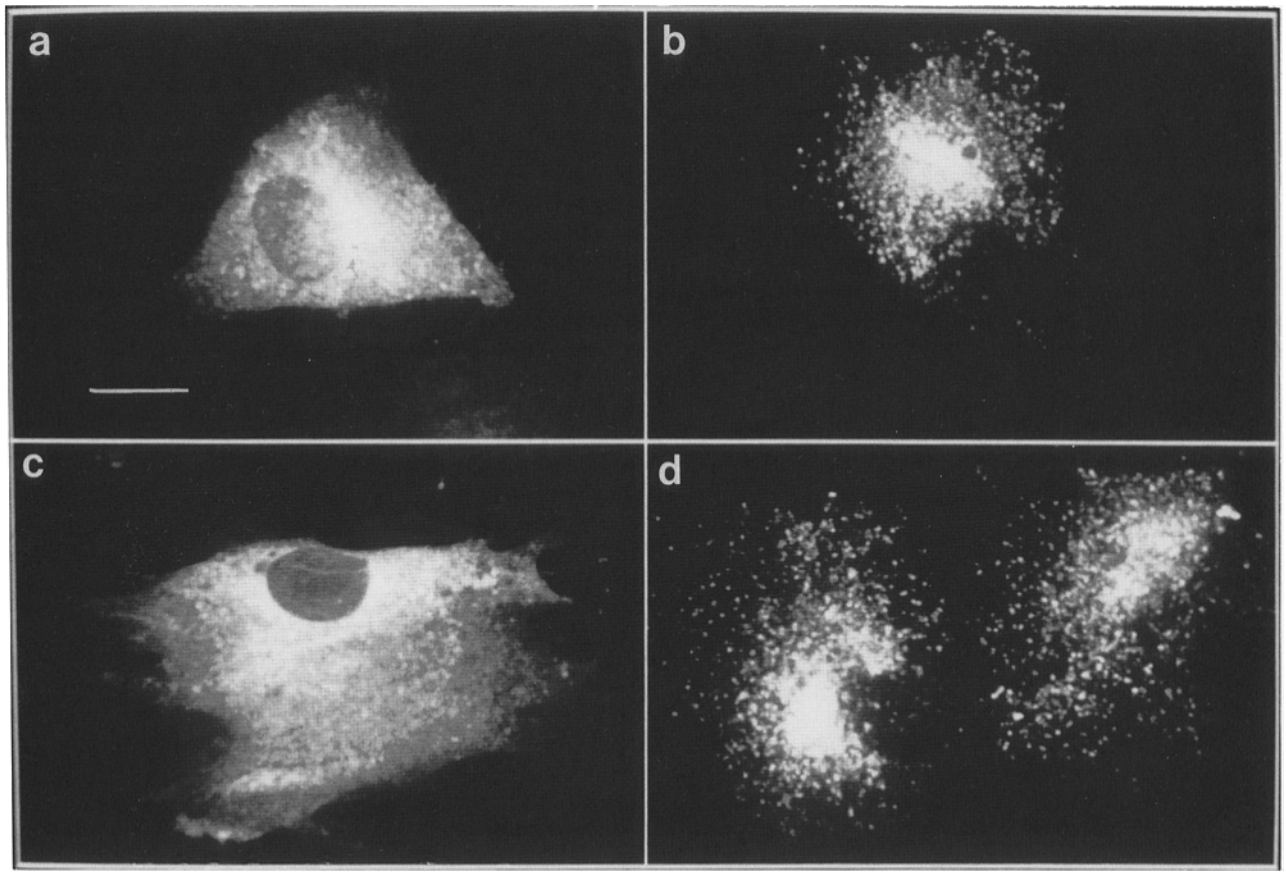

Figure 7. Localization of rhoA/rhoB chimeric proteins. Rat-2 cells microinjected with pEXV expressing cDNAs of myc-tagged $p21^{rho}$ proteins and stained after 16 h with 9E10 anti-myctag antibody. (*a*) $p21^{rhoA}$, (*b*) $p21^{rhoB}$, (*c*) $p21^{rhoB}$ (residues 1–178)$p21^{rhoA}$ (residues 179–193), (*d*) $p21^{rhoA}$ (residues 1–178)$p21^{rhoB}$ (residues 179–196). Bar, 10 μm.

rho proteins has been localized to the Golgi apparatus (McCaffrey et al., 1991) but it is not known if the function of RHO1 is similar to that of mammalian rho proteins. The localization of rhoB was quite different and distinct from that of rhoA and rhoC; intense fluorescence was observed in the early endosomal and prelysosomal compartment with a small fraction detectable at the plasma membrane. Colocalization of rhoB with both the prelysosomal marker, mannose-6-phosphate receptor, and with internalized transferrin but not with mature lysosomes confirmed this assignment of rhoB. The localization is consistent with the published purification of rhoB from a bovine brain membrane fraction (Hoshijima et al., 1990).

The signal sequences directing the ras proteins to their intracellular destination, the plasma membrane, have been characterized in great detail (Hancock et al., 1989, 1990, 1991). Two signals have been shown to be necessary, a COOH-terminal CAAX box and either a polybasic domain ($p21^{k\text{-}ras(B)}$) or palmitoylation of proximal cysteine residues ($p21^{Ha\text{-}ras}$ and $p21^{N\text{-}aas}$). These signals contain all the information required for plasma membrane localization since when they are fused to a heterologous protein they direct that protein to the plasma membrane (Hancock et al., 1991). The observed localization of the rho proteins was therefore somewhat unexpected since on the face of it they look very similar to ras at their carboxy termini. They each have a CAAX box, though for each rho, X = Leucine and as such it is expected to be geranylgeranylated and not farnesylated. This has already been confirmed in the case of rhoA (Katayama et al., 1991). In addition, rhoA and rhoC have basic amino-acid residues close to the CAAX box while rhoB has two upstream cysteines which are putative palmitoylation sites. Clearly, therefore, there must be additional signals determining the localizations of the rho proteins.

As a first step to delineate these sequences we fused the COOH terminus of rhoA (14 amino acids) and rhoB (17 amino acids) to a heterologous protein, protein A. In an analogous fashion to experiments with ras, the rhoB COOH terminal directed protein A exclusively to the plasma membrane. The rhoA COOH terminus, however, although able to associate with the plasma membrane still showed a significant amount in the cytosol. We conclude that the COOH terminus of rhoA has a weaker affinity for the plasma membrane than that of rhoB and therefore partitions predominantly into the cytosol. A comparison of the COOH termini of rhoA and K-ras(B) reveals that K-ras(B) has nine positive-charged residues while rhoA has five and it is possible that these differences define a weaker affinity for the plasma membrane. Indeed mutagenesis experiments on K-ras(B) have revealed that a reduction in the number of charged residues from 9 to 7 results in a marked increase in the amount of $p21^{K\text{-}ras(B)}$ in the cytosol (Hancock et al., 1991).

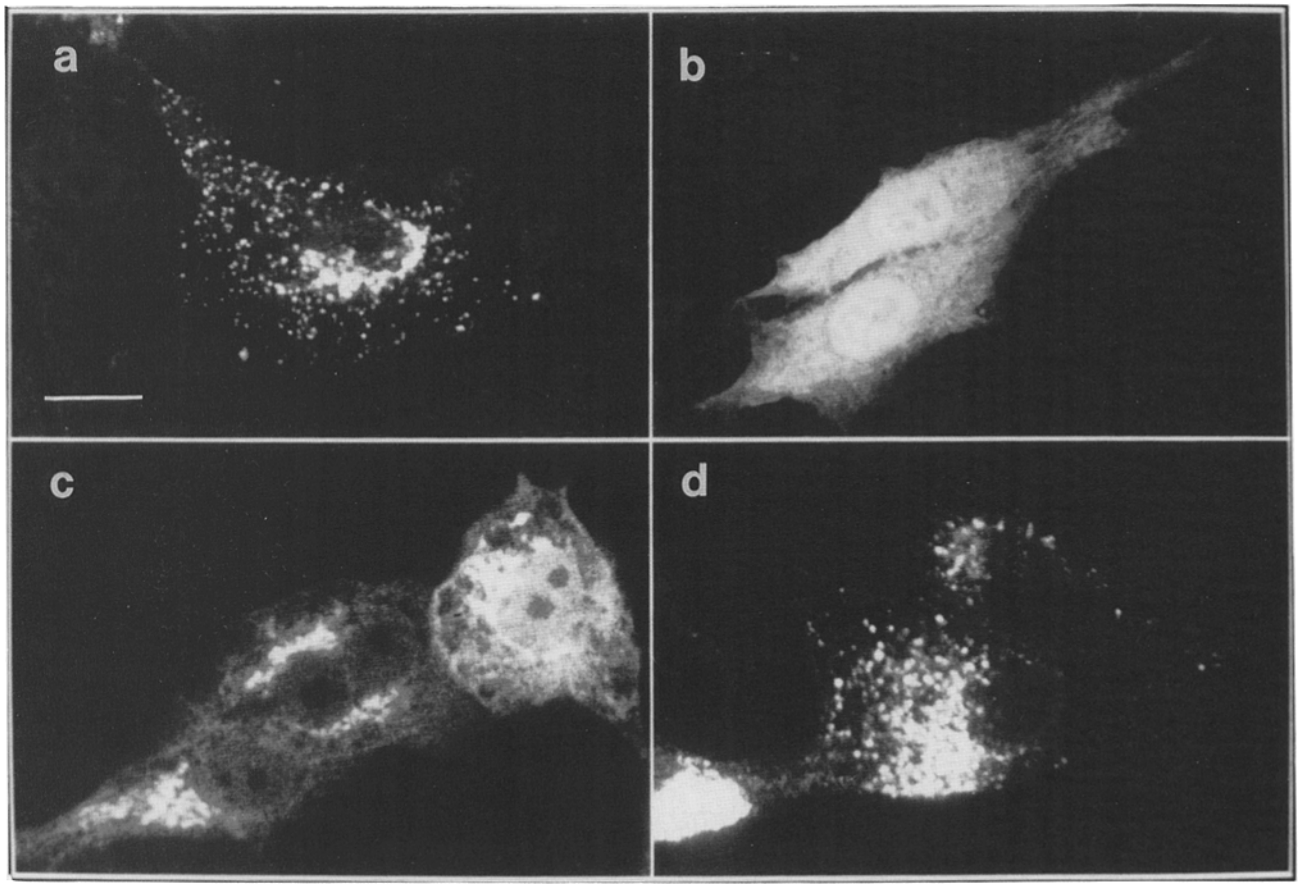

Figure 8. Localization of rhoB containing C-terminal mutations. Rat-2 cells microinjected with pEXV DNA (50 μg/ml) encoding myc-tagged rhoB cDNAs 16 h before staining with 9E10 mouse monoclonal anti-myctag antibody. (*a*) rhoB with unmutated COOH-terminus, (*b*) rhoB with cys193 to ser193 mutation, (*c*) rhoB with cys192 to ser192 mutation, (*d*) rhoB with cys189 to ser189 mutation. Bar, 10 μm.

The distribution of rhoB along the endosomal pathway, however, cannot be explained by the subtle variations in COOH-terminal sequence. In fact addition of even 136 amino acids of rhoB to protein A still resulted in a plasma membrane localization. We conclude that either the first 60 amino acids of rhoB are essential or that correct folding of the protein is required for recognition of signal sequence. To distinguish these two possibilities we constructed a chimera using the first 60 amino acids of N-ras and the remaining 136 amino acids of rhoB. This protein is capable of folding correctly and can bind GTP in vitro (our own unpublished observations). This chimera contains the effector region of ras and it is biologically active and capable of transforming cells almost as efficiently as oncogenic full-length N-ras. This chimera localized exclusively to the plasma membrane. We conclude therefore, that the first 60 amino acids of rhoB containing its putative effector region are essential for its localization. Finally to confirm these results we have constructed chimeras between rhoA and rhoB. When the COOH-terminal 14 amino acids of rhoA are replaced with the COOH-terminal 17 amino acids of rhoB the protein now localizes to the endosomal/prelysosomal compartment. Similarly, COOH-terminal rhoA sequences when attached to rhoB result in the protein being cytosolic like that of full-length $p21^{rhoA}$. This shows that only those sequences at the extreme COOH termini are responsible for the different intracellular localizations of rhoA and rhoB.

The identification of rhoA and rhoB in both a cytoplasmic intracellular compartment (cytosol or endosomal/prelysosomal vesicles) and in the plasma membrane suggests that they may be cycling between the two. We have shown that the COOH terminus of rhoB is capable of directing a heterologous protein to the plasma membrane but if the rho effector region is present, allowing the protein to interact with its target, it is directed to the endocytic pathway and internalized. RhoA on the other hand has only a weak affinity for the plasma membrane but shows increased plasma membrane association when the NH_2-terminal 60 amino acids are missing suggesting this sequence also contributes to the partitioning of rhoA predominantly to the cytosol. It is clear that the COOH-terminal cysteine within the CAAX box is important in defining the localization of the rho proteins as mutation leads to loss of the wild-type pattern of fluorescence. In addition, mutation of another cysteine residue in $p21^{rhoB}$ at codon 192, which may be a further site for posttranslational modification, also leads to changes in localization of the protein. The double cysteine motif at positions 192 and 193 makes the COOH-terminal structure of the protein more like that of the $p21^{rab}$ proteins (Balch, 1990 for review), and it has recently been shown that these COOH-terminal sequences within $p21^{rab}$ are essential in determining their correct localization (Chavrier et al., 1991). It may therefore be that $p21^{rhoB}$ is modified at its COOH terminus in a manner similar to that of some $p21^{rab}$ proteins rather that of $p21^{ras}$.

How does the localization of the rho proteins fit in with their biological function? Despite their different cellular locations microinjection of cells with recombinant rhoA or rhoB proteins produces an identical biological response; they both stimulate the assembly of actin stress fibers attached to adhesion plaques at the plasma membrane (Paterson et al., 1990; Ridley and Hall, 1992). Recent results in neutrophils have shown that two closely related proteins to rho, $p21^{rac1}$ and $p21^{rac2}$, are involved in promoting the assembly of cytosolic components onto a plasma membrane bound NADPH oxidase to regulate the production of superoxide (Abo et al., 1991; Knaus et al., 1991) but despite being clearly involved in this plasma membrane process, $p21^{rac}$ has been purified from the cytosol of neutrophils and macrophages. The mechanism by which the $p21^{rac}$ proteins cycle between cytosol and plasma membrane has not yet been investigated. By analogy it has been proposed that rho proteins are involved in regulating the assembly of cytosolic components at a plasma membrane target (Hall, 1992). After interacting with its target, rhoA can dissociate into the cytosol due to its intrinsic weak affinity for membranes. We have shown that the COOH-terminus of rhoB on the other hand enables it to enter the endocytic pathway for recycling but only after interacting with its target. Why the rho proteins should use different recycling pathways and the identity of the target molecules involved will require further analysis.

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